REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Final (15 Sep 94 - 14 Sep 99)

4. TITLE AND SUBTITLE
Egf Receptor Mabs and Chemotherapy/Characterization of Synergistic Interactions Between Cytotoxic Agents and Inhibitors of the Tyrosine Kinase Growth Factor Receptor Signaling Cascade

5. FUNDING NUMBERS
DAMD17-94-J-4329

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The focus of the laboratory and correlative science projects funded by this grant derive from the observation that antibodies directed against the EGFR receptor (225) and HER2 tyrosine kinase (4D5, trastuzumab) are effective in treating breast cancer in preclinical models. In the case of trastuzumab, these data have been extended to the treatment of human disease. Furthermore, Baselga and Mendelsohn and others have shown that inhibition of these tyrosine kinases with antireceptor antibodies augments the activity of a variety of cytotoxic agents. These results have been validated in large, prospective clinical trials, in which trastuzumab has meaningfully improved survival for patients with HER2/neu overexpressing metastatic breast cancer when combined with paclitaxel or doxorubicin + cyclophosphamide. This report describes that drugs that inhibit other elements in the HER-kinase signalling pathway also inhibit breast cancer cells, and determines whether such inhibitors synergize with taxanes, to better elucidate the function of HER2 in breast cancer. Our investigation of the immunophenotypic characterization of human breast cancers for molecular markers thought to be potential correlates of the antitumor activity of taxanes, examining HER2 among other candidates, is similarly relevant to optimizing and individualizing anti-cancer treatment.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES 18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

AD_____

Award Number: DAMD17-94-J-4329

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REPORT DATE: October 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
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Introduction

Our work supported by this grant builds on the observation that antibodies directed against the EGFR receptor (225) and HER2 tyrosine kinase (4D5, trastuzumab) are effective in treating breast cancer in preclinical models. In the case of trastuzumab, these data have been extended to the treatment of human disease. Furthermore, Baselga and Mendelsohn and others have shown that inhibition of these tyrosine kinases with antireceptor antibodies augments the activity of a variety of cytotoxic agents. These results have been validated in large, prospective clinical trials, in which trastuzumab has meaningfully improved survival for patients with HER2/neu overexpressing metastatic breast cancer when combined with paclitaxel or doxorubicin + cyclophosphamide.

The goals of these studies were to determine whether drugs that inhibit other elements in the HER-kinase signalling pathway also inhibit breast cancer cells, to determine whether such inhibitors synergize with taxanes, and to use these studies to begin to elucidate the function of HER2 in breast cancer. Furthermore, an investigation of the immunophenotypic characterization of human breast cancers for molecular markers thought to be potential correlates of the antitumor activity of taxanes was undertaken, examining HER2 among other candidates.

1. Studies on farnesyl transferase inhibitors (FTI's): HER kinases mediate their effects through several signalling pathways, including those regulated by Ras, PI3 kinase, and Stat activation. The Ras-MAP kinase pathway is a key effector of most growth factor signals, and Ras is mutated in approximately 30% of human cancers. Although Ras is rarely, if ever, mutated in breast cancer, activation of wild type Ras by activated tyrosine kinases is felt to be necessary for the growth of many breast cancers.

   The Ras protein is prenylated with a fifteen carbon farnesyl group and this post-translational modification is required for its localization in the membrane and for the transforming ability of mutated Ras. Farnesylation is catalyzed by a specific farnesyl transferase and it was reasoned that a farnesyl transferase inhibitor (FTI) would prevent Ras processing in a fairly selective manner and effectively inhibit the growth of cells that required Ras for transformation.

   Many pharmaceutical companies have now developed potent and specific FTIs. Although there drugs were developed as agents for the treatment of tumors harboring Ras mutation, we reasoned that if they were effective in inhibiting the processing of wild-type Ras, they might be useful therapeutics for breast cancer.

1.A. Effects of farnesyl transferase inhibitors on breast cancer cells

We found that FTIs inhibited the anchorage-dependent and independent growth of most human cancer cell lines, including many breast cancer cell lines. Inhibition was independent of the presence of mutated Ras. Some breast cancer cell lines (MCF-7, MDA-468) were among the most sensitive to the drug. FTI inhibited the processing of wild type H-Ras. Despite this, it was not particularly toxic in animals (or human phase I studies, *vide infra*). FTI did not inhibit the processing of mutated or wild type K-Ras in breast or other cancers. We did show that some tumor cell lines with mutated K-Ras were sensitive to FTI, even though the K-Ras protein remained processed.

These and other data suggested to us that FTI worked by multiple mechanisms, some of which are Ras-independent. We set out to determine the mechanism in breast cancer cells. We found that some breast cancer cells, but not most, arrested in the G1 phase of the cell cycle when exposed to FTI. We showed that G1 block only occurred in cells that have wild type p53 and is due to p53 dependent induction of the p21 cyclin kinase inhibitors. Inactivation of p53 or p21 abrogates the effect of FTI on G1 cyclin kinases. Instead, the cells undergo endoreduplication and then apoptosis in mitosis.

We have thus uncovered three mechanisms of FTI action. FTI inhibits cancer cells that require functional, mutated H-Ras by inhibiting its processing. It induces p21 and causes G1 block in cells with wild type p53. In some tumors with mutated p53 it

causes death in mitosis. Since approximately 70% of breast cancers have wild type p53, this mechanism would seem particularly apposite in this disease. We asked whether the inhibitory effect of FTI on breast cancers with wild type p53 and Ras is due to inhibition of Ras processing. MCF-7 is an estrogen receptor containing breast cancer cell line with wild type p53 and Ras that is quite sensitive to FTI. At FTI concentrations that induce p21 and cause G1 arrest, neither Ras processing or signalling function are affected. Furthermore, introduction of high levels of the N17-dominant negative Ras in this cell result in inhibition of Ras signalling, but not of anchorage independent growth.

These data suggest that functional Ras is not required for the growth of at least some breast cancers, and that FTIs work in these cancers by Ras independent mechanisms. They also imply that FTIs may be useful in a subset of human breast cancers.

1.B. FTIs in combination with Mo Ab 225

FTI does inhibit H-Ras processing. This suggests the possibility that it might act in concert with antibodies that inhibit the activation of receptor tyrosine kinases. Furthermore, FTI has been relatively non-toxic in phase I and II clinical trials, so it might be a useful agent to administer in combination. MCF10A is an immortalized but non-transformed human mammary epithelial cell line. It is EGF-dependent and quite sensitive to inhibition by the anti-EGFr antibody, 225. MoAb 225 causes MCF10A to arrest in the G1 phase of the cell cycle. G1 arrest is associated with induction of p27kip. On the other hand, MCF10 is relatively resistant to FTI, and is inhibited only at fairly high concentrations.

MCF10A can be transformed by mutated, activated H-Ras. These transformants are no longer EGF-dependent and they are only slightly inhibited by 225. In contrast, the H-Ras-MCF10A cells are sensitized to FTI. FTI inhibits their growth at low concentrations, but does not cause G1 arrest. However, the combination of 225 and FTI synergistically inhibits this cell line and causes G1 arrest. Exposure of MCF10A-H-Ras to the combination results in synergistic induction of p27. The results suggest that activation of EGF receptor tyrosine kinase affects G1 progression by Ras-dependent and Ras-independent mechanisms. They also provide some evidence that this combination of inhibitors might be useful clinically, at least in tumors that harbor mutated H-Ras and an active transmembrane tyrosine kinase. Whether it might also be useful in breast cancer is unclear. It is possible that in tumors that co-express an activated HER kinase and high levels of wild type H-Ras, inhibition of both would be useful.

1.C. FTIs in combination with cytotoxics

As suggested above, the low toxicity of FTI and novel mechanism make it a logical candidate for inclusion in combination therapeutic regimens. We showed that, in

tissue culture, doxorubicin, vincristine, cisplatinum, and other agents have additive antitumor activity when administered with FTI. These data suggest that FTI will not interfere with the actions of traditional cytotoxics and therefore may be useful in combination.

Of greater interest, taxanes and FTI synergized in several breast cancer cell lines. This synergy does not seem to be the result of effects on drug metabolism or transport. Rather, FTI sensitizes the mitotic checkpoint to taxanes. In cell lines in which FTI has no effect by itself on mitotic progression, it synergizes with taxanes to cause mitotic arrest. This effect is not generally seen with agents that activate the spindle checkpoint. Nocodazole, vincristine, and vinblastine, drugs that prevent spindle formation, do not synergize with FTI. **Epothilones** and taxanes, agents that prevent microtubule depolymerization, **do** synergize. Thus, this phenomenon distinguishes between activation of the checkpoint-mediated failure to form a spindle and that caused by am inability to dissemble the spindle.

The phenomenon of FTI-taxane synergy has been reproduced in multiple animal models by investigators at Schering-Plough and other drug companies. These data now form the basis for several clinical trials, including one that we have intiated at MSKCC.

2. Studies on Ansamycin Antibiotics

Motivated by the effects observed with FTI, 225, epothilone, and taxanes as described above vis a vis the perturbation of growth factor receptor signalling, we were motivated to study another class of drugs that work in part, by inducing the destruction of HER-family tyrosine kinases. Ansamycins are natural products that bind to a conserved pocket in the protein chaperone hsp90. This changes hsp90 function such that the signal proteins that require hsp90 for conformational maturation are, instead, degraded in the proteasome. Exposure of cells to ansamycins results in the degradation of steroid receptors, Raf serine kinase, and certain transmembrane tyrosine kinases. We and others have found that HER2, EGFr, and the met tyrosine kinases are among the most sensitive targets of the drug.

One might expect that inhibition of the general chaperone function of hsp90 and degradation of so many important signal proteins would have non-specific toxic effects on cells. This is not the case. We showed that ansamycins such as geldanamycin (GM) and herbimycin A cause rapid down regulation of cyclin D-associated kinase activity and an Rb-dependent G1 block. Cells with mutated Rb do not block in G1. Instead, they arrest in prometaphase and undergo apoptotic death. These results suggest that inhibition of hsp90 function by ansamycins selectively affects pathways upstream of Rb. We have gone on to show that the effect on cyclin D-associated kinase is secondary to down regulation of a PI3-kinase, akt kinase regulated pathway that is required for D-cyclin translation.

Breast cancer cells that overexpress HER2 are especially sensitive to GM (IC50 5-10 nM). Sensitivity is associated with a rapid loss in akt activity and D-cyclin expression. We are currently investigating the mechanism of loss of akt activity. Our hypothesis, supported by preliminary data, is that overexpression of HER2 in cells that express HER3 results in high levels of PI3 kinase and akt kinase activity with a concomitant increase in D-cyclin expression. GM causes HER2 degradation, down regulation of the pathway, and loss of D-cyclin expression. We believe that one of the primary effects of HER2 amplification in breast cancer is activation of this pathway, which results in deregulated G1 progression and cellular resistance to apoptosis. Addition of GM to HER2-overexpressing breast cancer cells causes G1 arrest and subsequent differentiation, followed by apoptosis.

The inhibition of akt kinase by GM suggests that it might cause synergistic apoptosis when administered with cytotoxic drugs. Indeed, our preliminary data shows that GM synergizes with both taxanes and doxorubicin. The taxane synergy is schedule dependent. Prior administration of GM causes G1 block and prevents induction of apoptosis by paclitaxel; administration of GM at the same time of after paclitaxel causes synergistic apoptosis. As predicted, the synergy is schedule independent in cells deficient in Rb function and for doxorubicin in all cells.

The experiments have important clinical implications. They suggest that the trastuzumab-chemotherapy synergy may be mediated by inhibition of aky by trastuzumab. Experiments are now in progress to ascertain whether this is so. They also suggest that GM and cytotoxic chemotherapy might be clinically useful. A derivative of GM, 17-allylaminogeldanamycin (17-AAG) in now in phase I clinical trial at our institution and elsewhere. This trial is sponsored by the NCI. We have shown that 17-AAG is active in xenograft models of breast cancers with elevated HER2 expression, that drug activity is associated with rapid loss on HER2 expression and akt activity, and that the combination of paclitaxel and 17-AAG is much more active than either agent alone.

3. Characterization of molecular immunophenotype of pretreatment breast cancer tissue for association with clinical taxane response, and with response to taxane + trastuzumab

3.A. Immunophenotypic Characterization of Breast Cancers for Association with Clinical Taxane Response:

The taxanes, paclitaxel and docetaxel are arguable the most effective chemotherapeutic agents against breast cancer. They cause cellular structures known as microtubules to polymerize, or form a network, and stabilize this lattice against the forces that normally destabilize it. The net result of this effect is the disruption of

normal cell division (mitosis) with arrest of cells at the G2-M interphase of the cell cycle.

The cell cycle is governed by a family of enzymes known as cyclin-dependent kinases (cdks), which are regulated by associated molecules known as cyclins, and by chemical phosphorylation. Distinct from some other chemotherapy drugs, taxanes have been reported to induce programmed cell death (apoptosis) mediated by a pathway that appears independent of the tumor suppressor p53. A checkpoint blockade at the G1/S boundary of the cell cycle would be expected to promote taxane-induced apoptotic cell death. P27kip, a cdk inhibitor, regulates progression from G1 into S phase by binding and inhibiting cyclins/CDKs. Lower p27 levels in breast cancers assessed by immunohistochemistry have been associated with shorter survival (Porter et al. Nature Med 3:222-5, 1997, and Tan et al. Cancer Res 57:1259-63, 1997). Furthermore, increased p27kip expression has been observed in pacliaxel-resistant 435/to.3 breast cancer cells compared to its parental MDA 435 cell line (St. Croix et al. Nature Med 2:1204-10, 1996).

We have previously examined the possible association of overexpression of HER2 with clinical taxane sensitivity in a preliminary study of tumor tissue from women receiving either paclitaxel or docetaxel as monotherapy for metastatic breast cancer. Multivariate analysis revealed that HER2 overexpression as assessed by the monoclonal antibody 4D5 (the murine homologue of trastuzumab, or Herceptin) was associated with a significantly increased likelihood of clinical response to single agent taxane (Baselga et al. Oncology 11:43-8, 1997). We also performed the same multivariate analysis evaluating HER2 by a different immunohistochemical (IHC) antibody, a polyclonal antibody known as pAb-1. With this antibody, which targets a different epitope, and may lack sufficient specificity for the HER2 receptor, no relationship between HER2 expression and taxane response was discerned. This "immunohistoconfusion" prompted further detailed characterization of HER2 status in a larger data set.

We performed IHC analysis of breast cancer tissue for ER, PR, EGFr, HER2, p53, bcl-2, bax, and p27 on 144 paraffin-embedded tumor tissues available from patients treated on any one of a series of 9 IRB-approved phase II clinical trials conducted at our institution of either paclitaxel or docetaxel as monotherapy for metastatic breast cancer. 100 blocks were from primary tumor sites, 40 from metastatic sites, with 9 of this latter group obtained from chemotherapy-naiive patients. Patient demographic characteristics that could conceivably influence response, such as performance status, extent of metastatic disease, and prior anthracycline exposure were also examined. In univariate chi squared analysis, Karnofsky Performance status ($p=.003$) and to a lesser extent, prior anthracycline exposure ($p=.041$) were significant demographic predictors of the likelihood of good taxane response for metastatic disease. Of the molecular markers analyzed, only lack of p27 expression approached significance ($p=.075$) as a correlate of clinical taxane sensitivity. We performed a multivariate analysis of the associateion of p27 status and taxane response adjusting for the potential confounding clinical variables "extent of disease", "prior anthracycline" and

"Karnofsky Performance Status (KPS)" and found that while there was a trend toward the independent significance of lack of p27 expression as a predictor of taxane response (e.g. Mantel Haenszel Chi Squared p=.068, with an odds ratio of taxane response = 1.88 (95% CI 0.95-3.69), controlling for KPS. Given the hypothesis that one possible mechanism for the clinical therapeutic effect of taxanes is the promotion of apoptosis at the G1 checkpoint, we evaluated p53, bcl-2 and bax: none were associated with clinical taxane response.

We chose to either confirm or refute our prior observation in a smaller dataset that HER2 status might be a predictive factor for clinical taxane sensitivity. On a larger sample, with many new blocks/specimens retrieved vis a vis our prior analysis, we chose to evaluate HER2 status by two antibodies. One was the **monoclonal** antibody CB11 (Ventana, Inc.), particularly since this IHC test was part of the "CTA" (Clinical Trials Assay) used to select patients for Genentech's prior Herceptin clinical trials. The second was the **polyclonal** HercepTest™ (Dako, Inc.), as this assay was FDA-approved for clinical use in 1998. Our present analysis shows that *neither* antibody, CB11 (p=.192) nor HercepTest™ (p=.511) was associated with clinical taxane response. We plan to further analyze these specimens for HER2 gene amplification with the PathVysion™ (Vysis, Inc.) fluorescent in situ hybridization (FISH) assay. This work has motivated a larger study within the Correlative Science Committee of the Cancer and Leukemia Group B.

3.B. Immunophenotypic and Genotypic Characterization of HER2 and Association with Clinical Response to Taxane + Trastuzumab

As previously noted, the combination of paclitaxel (administered every 3 weeks) together with trastuzumab (weekly) has been shown to exhibit impressive preclinical antitumor activity (Baselga et al. Cancer Res 58:2825-31, 1998), and the combination has prolonged survival for women with HER2-overexpressing metastatic breast cancer as compared to paclitaxel alone (Slamon et al. Proc ASCO 1998, Norton et al. Proc ASCO 1999). We were motivated to perform a large phase II clinical trial exploring the potential that the weekly co-administration of both paclitaxel and trastuzumab based on these factors, coupled with our own demonstration of the efficacy and safety of weekly paclitaxel infusions (Seidman et al. J Clin Oncol 16:3353-61, 1998). In the context of this trial, which accrued 95 patients, we endeavored to characterize HER2 status using 4 different IHC antibodies, and to simultaneously assess HER2 gene amplification by FISH. Correlation with clinical response to the weekly regimen of paclitaxel + trastuzumab was performed. While a reasonably high level of concordance between monoclonal antibodies (CB11 and TAB250) and polyclonal antibodies (HercepTest and p-Ab1) was observed, 21% of patient's tumor tissues tested positively by polyclonal antibody, but negatively by monoclonal antibody. This discordant group of patients had a significantly lower likelihood of response to therapy than the group of patients whose tumors overexpressed HER2 by *both* monoclonal and polyclonal antibody (40% vs. 75.6%, p=.01). Monoclonal antibodies TAB250 (p=.001) and CB11 (p=.006) were more

optimal predictors of clinical response to this therapy than were the polyclonal antibodies HercepTest (p=.059) and p-Ab1 (p=0.05), as was FISH (Vysis), p=.01.

This work, together with the work of a limited number of other investigators, has has real and immediate practical impact, as it has prompted a reassessment of HER2 testing in the selection of patients. Indeed, clinical trial eligibility criteria are being modified accordingly to as to better select those patients most likely to derive clinical benefit from trastuzumab-based therapies.

KEY RESEARCH ACCOMPLISHMENTS

- **Characterization of anticancer mechanism of action of farnesyl transferase inhibitors (FTIs) in breast cancer cells**

- **Demonstration of synergistic antitumor activity of farnesyl transferase inhibitor and epidermal growth factor receptor directed monoclonal antibody in H-Ras-MCF10A breast cancer cells**

- **Elucidation of cell cycle effects of additive and synergistic combinations of FTI and cytotoxic chemotherapeutic agents in preclinical models of breast cancer**

- **Definition of 17-allyl, amino geldanamycin's (an ansamycin) effect on inducing the destruction of HER-family tyrosine kinases. This novel HER2-directed strategy has been "translated", and is being studied presently in a human clinical trial at MSKCC**

- **Performance of multivariate analysis of potential clinical and immunophenotypic molecular correlates of clinical sensitivity to taxane monotherapy for metastatic breast cancer, including HER2, p53, bcl2, bax, p27**

- **Demonstration that monoclonal antibodies directed at HER2, and fluorescent *in situ* hybridization testing for HER2 gene amplification are better predictors of clinical response to paclitaxel + trastuzumab (Herceptin) therapy for metastatic breast cancer.**

REPORTABLE OUTCOMES

Abstract presentations:

1. **2000 Era of Hope Meeting, Atlanta, Georgia**
2. **1999, 2000, and 2001 American Society of Clinical Oncology Meetings**
3. **1999 San Antonio Breast Cancer Symposium (Breast Ca Res Treat 57 (1):29)**

Manuscripts:

In preparation, for submission to J Clin Oncol, Cancer Research

Patent and Licenses Applied for or Issued: None

Degrees obtained that are supported by this award: None

Development of Cell Lines, Tissue, or Serum Repositories: Tumor tissue bank as described in Section 3 of Body of report (*vide infra)*

Informatics: None

Funding Applied for Based on Work Supported By this Award: None Yet

Employment or Research Opportunities Applied for and/or Received on Experiences/Training Supported by this Award:

Dr. Laura Sepp-Lorenzino (trained in Dr. Rosen's lab): Senior Investigator, Merck Pharmaceutical Research Institute

CONCLUSIONS

The results of our laboratory and correlative investigations characterizing the interactions between cytotoxic agents and inhibitors of the tyrosine kinase growth factor receptor signalling cascade are of importance and highly relevant both from a basic science perspective (unravelling the cellular effects that lead to maximal antitumor activity upon exposure to novel agents such as farnesyl transferase inhibitors, tyrosine kinase growth factor directed monoclonal antibodies, geldanamycin, etc.), and from a clinical vantage point: similar to the manner in which earlier work by Baselga and Mendelsohn with paclitaxel and 4D5 motivated a multinational prospective randomized trial that led to the discovery of prolonged survival for trastuzumab and paclitaxel, data derived from the work supported by this Department of Defense grant have already driven the design of ongoing translational clinical trials at our institution. Specifically, the clinical evaluation of 17 allyl, amino geldanamycin, and the combination of paclitaxel and a farnesyl transferase inhibitor are ongoing.

Our investigation of molecular correlates of clinical sensitivity to taxane monotherapy has driven the design of a correlative science project currently being performed on a national basis, as a companion study to Cancer and Leukemia Group B Clinical Trial 9840. This trial addresses the characterization of HER2 "status" (phenotype/genotype) in an attempt to get beyond the "immunohistoconfusion" that has existed regarding the optimal selection of patients for Herceptin-based (or indeed any HER2-directed) breast cancer therapy. The preliminary observation of p27 as a correlate of clinical sensitivity to taxane therapy is presently being studied on a larger sample size, pending the availability of additional tumor tissue for analysis.

REFERENCES (included within Body)